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Article

Hyperinsulinemia Drives Diet-Induced Obesity Independently of Brain Insulin Production

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SUMMARY

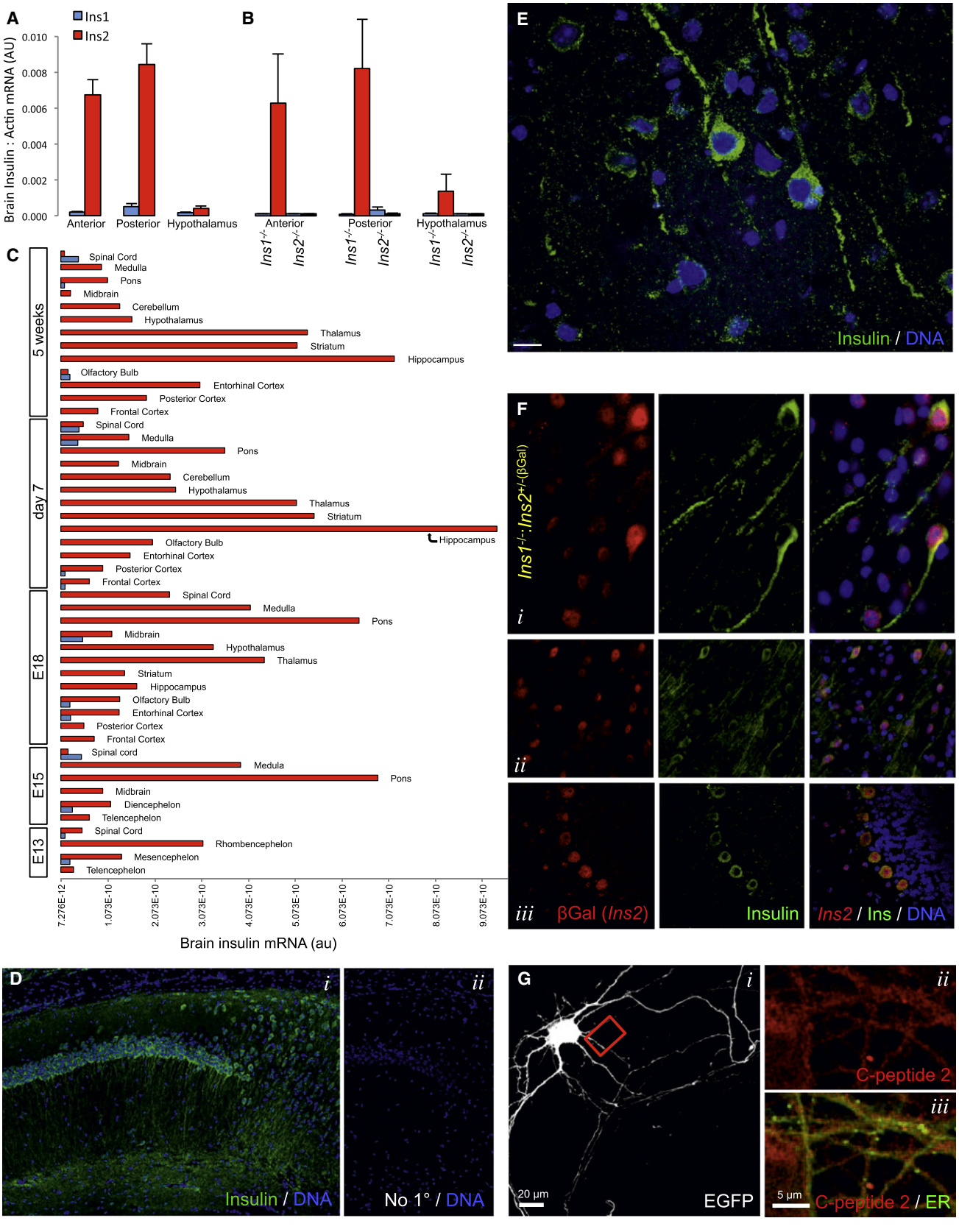
Hyperinsulinemia is associated with obesity and pancreatic islet hyperplasia, but whether insulin causes these phenomena or is a compensatory response has remained unsettled for decades. We examined the role of insulin hypersecretion in diet-induced obesity by varying the pancreas-specific *Ins1* gene dosage in mice lacking *Ins2* gene expression in the pancreas, thymus, and brain. Age-dependent increases in fasting insulin and β cell mass were absent in $Ins1^{+/-}$:$Ins2^{-/-}$ mice fed a high-fat diet when compared to $Ins1^{+/+}$:$Ins2^{-/-}$ littermate controls. Remarkably, $Ins1^{+/-}$:$Ins2^{-/-}$ mice were completely protected from diet-induced obesity. Genetic prevention of chronic hyperinsulinemia in this model reprogrammed white adipose tissue to express uncoupling protein 1 and increase energy expenditure. Normalization of adipocyte size and activation of energy expenditure genes in white adipose tissue was associated with reduced inflammation, reduced fatty acid spillover, and reduced hepatic steatosis. Thus, we provide genetic evidence that pathological circulating hyperinsulinemia drives diet-induced obesity and its complications.

INTRODUCTION

Obesity is a worldwide epidemic with increasing prevalence and is a major independent risk factor for heart disease, hypertension, stroke, diabetes, cancer, and many other diseases (Després and Lemieux, 2006; Olshansky et al., 2005). The molecular causes of obesity remain largely unexplained, despite the identification of common gene variants that contribute modest risk (Frayling et al., 2007). Obesity research has benefited from animal models in which genetic and environmental factors can be manipulated in ways that are impossible with humans. For example, invertebrates with reduced insulin or insulin signaling have leaner, smaller bodies, along with increased life span (Broughton et al., 2008; Li et al., 2003). Studies in mammals point to cell type-specific roles for the insulin receptor in diet-induced obesity. Fat-specific insulin receptor knockout mice are protected from diet-induced obesity and have extended longevity (Blüher et al., 2002; Katic et al., 2007), whereas elimination of insulin receptors from some, but not all, brain regions increases food intake and obesity (Brüning et al., 2000; Könner and Brüning, 2012). The interpretation of studies involving insulin receptor ablation is confounded by compensation between tissues and disruption of insulin-like growth factor signaling involving hybrid receptors (Blüher et al., 2002; Kitamura et al., 2003). Moreover, knockout of the insulin receptor, by definition, induces tissue-specific insulin resistance, which alone may promote hyperglycemia and insulin hypersecretion (Brüning et al., 1998; Kitamura et al., 2003). Previous studies have been unable to determine the role of hyperinsulinemia on obesity in the absence of insulin resistance and glucose intolerance.

It is a widely held view that hyperinsulinemia simply represents a compensation for systemic insulin resistance. Nevertheless, the cause-and-effect relationships between hyperinsulinemia, insulin resistance, obesity, and type 2 diabetes remain unresolved. Reports of elevated insulin secretion at birth, and of insulin hypersecretion preceding obesity or insulin resistance, are inconsistent with hyperinsulinemia being merely an adaptive response (Genuth et al., 1971; Gray et al., 2010; Ishikawa et al., 1998; Kitamura et al., 2003; Koopmans et al., 1997; Le Stunff and Bougnères, 1994; Lustig, 2006; Odeleye et al., 1997; Shanik et al., 2008; Zimmet et al., 1991). Furthermore, blocking hyperinsulinemia with drugs including diazoxide or octreotide can cause weight loss in humans (Alemzadeh et al., 1998; Lustig et al., 2006), although interpretation of these studies is complicated by direct effects of these treatments on multiple tissues, including white fat (Shi et al., 1999) and brain (Pocai et al., 2005). Thus, although circumstantial evidence points to a role for hyperinsulinemia in the development of obesity, to date, there has been no direct evidence that circulating insulin itself drives obesity in mammals. To directly test this hypothesis, one would ideally genetically lower the amount of pancreatic insulin available without crossing the threshold that would result in diabetes.

Members of the insulin gene family are evolutionarily conserved products of neurosecretory cells (Broughton et al., 2008; Li et al., 2003). In mammals, insulin is recognized for its critical metabolic roles, whereas the insulin-like growth factors are most often associated with tissue growth. Evidence from our group and others points to important growth factor roles of insulin (Blüher et al., 2002; Johnson and Alejandro, 2008; Okada


A
Ins1
Ins2
Brain Insulin : Actin mRNA (AU)
0.010
0.008
0.006
0.004
0.002
0.000
Anterior
Posterior
Hypothalamus
B
Anterior
Posterior
Hypothalamus
Ins1-/-
Ins2-/-
C
5 weeks
day 7
E18
E15
E13
Spinal Cord
Medulla
Pons
Midbrain
Cerebellum
Hypothalamus
Thalamus
Striatum
Hippocampus
Olfactory Bulb
Entorhinal Cortex
Posterior Cortex
Frontal Cortex
Spinal cord
Medula
Diencephelon
Telencephelon
Rhombencephelon
Mesencephelon
7.276E-12
1.073E-10
2.073E-10
3.073E-10
4.073E-10
5.073E-10
6.073E-10
7.073E-10
8.073E-10
9.073E-10
Brain insulin mRNA (au)
E
Insulin / DNA
F
Ins1-/-:Ins2+/-(βGal)
i
ii
iii
βGal (Ins2)
Insulin
Ins2 / Ins / DNA
D
i
Insulin / DNA
ii
No 1° / DNA
G
i
20 μm
EGFP
ii
C-peptide 2
iii
5 μm
C-peptide 2 / ER

et al., 2007). Mice and rats have two insulin genes, producing hormones with similar peptide sequences. Deletion of both rodent insulin genes causes death from neonatal diabetes (Duvillié et al., 1997). It has been reported that ablation of either *Ins1* or *Ins2* from the mouse genome does not result in significant changes in glucose homeostasis in young mice fed a normal diet, implying compensation or redundancy with regards to metabolism (Duvillié et al., 1997; Leroux et al., 2001). However, there is evidence that the two rodent insulin genes have specific tissue distribution patterns suggesting some nonoverlapping functions (Kojima et al., 2004; Moore et al., 2001; Pugliese et al., 1997). Most studies have shown that *Ins1* is restricted to pancreatic β cells, where it contributes to approximately one-third of the expressed and secreted insulin (Hay and Docherty, 2006). *Ins2* is the ancestral gene, with gene structure, parental imprinting, and a broad tissue distribution similar to human *INS* (Deltour et al., 1993; Hay and Docherty, 2006).

In the present study, we took advantage of the pancreas-specific expression of the murine *Ins1* gene to demonstrate that diet-induced pancreatic insulin hypersecretion promotes obesity and its associated complications in this model. Mice made genetically incapable of high-fat diet-induced fasting hyperinsulinemia had increased levels of Ucp1 in white adipose tissue and increased energy expenditure. These results have profound implications for our understanding of the role of insulin in obesity and reveal unappreciated in vivo connections between fasting hyperinsulinemia and adipocyte reprogramming. As the human *INS* gene is subject to genetic variation (Le Stunff et al., 2001), our results suggest a mechanism for establishing obesity susceptibility to dietary stress.

RESULTS

Ins2, but Not Ins1, Is Expressed in the Central Nervous System

While it is clear that pancreatic β cells are the only significant source of circulating hormonal insulin, it is known that some cells outside the pancreas express modest amounts of *Ins2/INS.* For example, *Ins2* production in the thymus is widely accepted to play a role in immune tolerance (Fan et al., 2010; Pugliese et al., 1997). Over 80 reports have suggested that insulin can be produced in the brain (de la Monte and Wands, 2008; Deltour et al., 1993; Devaskar et al., 1993; Giddings et al., 1985; Havrankova et al., 1978; Lamotte et al., 2004; Wicksteed et al., 2010) (see the Supplemental Information available online). Deep-sequencing data from over 200 tissue libraries revealed broad expression of *Ins2*, but not *Ins1*, in multiple regions of the developing and postnatal brain (Figure S1A). The observation of central nervous system (CNS) *Ins2* prompted us to employ Taqman quantitative PCR (qPCR) primers, validated in $Ins2^{-/-}$ or $Ins1^{-/-}$ islets (Figure S2A), to elucidate the expression pattern of insulin in the brain. We observed brain expression of *Ins2* messenger RNA (mRNA), significantly above the background seen in $Ins2^{-/-}$ brain tissue (Figures 1A and 1B). *Ins1* mRNA was absent according to these criteria. Using an array with complementary DNA (cDNA) from multiple brain regions, we found *Ins2* was broadly expressed (most prominently in the hippocampus), while *Ins1* was virtually absent (Figure 1C). Consistent with a local role for neuronal insulin, quantitative analysis (Figure S2B) confirmed that *Ins2* expression in hippocampal neurons is much lower than in pancreatic islets, which must deliver insulin to the entire body. A similar RT-qPCR analysis of human brain cDNA array confirmed *INS* expression in several regions, including hippocampus (Figure S1B). Chromatin immunoprecipitation with antibodies to methylated histones, marking regions of active transcription, followed by direct sequencing pointed to active transcription around the *Ins2* gene, but not the *Ins1* gene, in cerebral cortex and cerebellum (Figure S1C). Both genes had activity marks in pancreatic islets (Figure S1C). Interestingly, the active regions near the *Ins2* locus in brain appear to be distinct from those in islets. These observations are consistent with many reports that *Ins2* promoters, but not *Ins1* promoters, exhibit robust activity in the brain (e.g., Wicksteed et al., 2010).

We studied the localization of insulin protein throughout the brain using antibodies validated in $Ins2^{-/-}$ or $Ins1^{-/-}$ islets and $Ins1^{-/-}$:$Ins2^{-/-}$ neurons (Figure S2C–S2E). Specific insulin immunoreactivity and C-peptide immunoreactivity were observed in the hippocampus, anterior olfactory nucleus, cerebral cortex, cerebellar Purkinje neurons, and other discrete nuclei (Figures 1D–1G). We also took advantage of the fact that the $Ins2^{-/-}$ mice used in our studies had LacZ knocked into in their endogenous *Ins2* locus (Figure S2E). We found that the same adult neurons that were positive for βGal also stained robustly for insulin protein in $Ins1^{-/-}$:$Ins2^{+/-(\beta gal)}$ mice (Figure 1F). To rule

Figure 1. Central Nervous System Expression of *Ins2*, but Not *Ins1*

(A) Analysis of *Ins2* and *Ins1* mRNA expression (relative to the control gene) in the hypothalamus, posterior brain, and anterior brain of 6-month-old wild-type C57Bl6/J mice.

(B) No significant compensatory upregulation of either insulin gene in $Ins1^{-/-}$:$Ins2^{+/+}$ or $Ins1^{+/+}$:$Ins2^{-/-}$ mice. These mice also provide ideal negative controls for the qRT-PCR. Further validation of the specificity of mouse *Ins1* and *Ins2* Taqman real-time qPCR primer/probe sets in *Ins1* or *Ins2* knockout mouse islets can be found in the Supplemental Information.

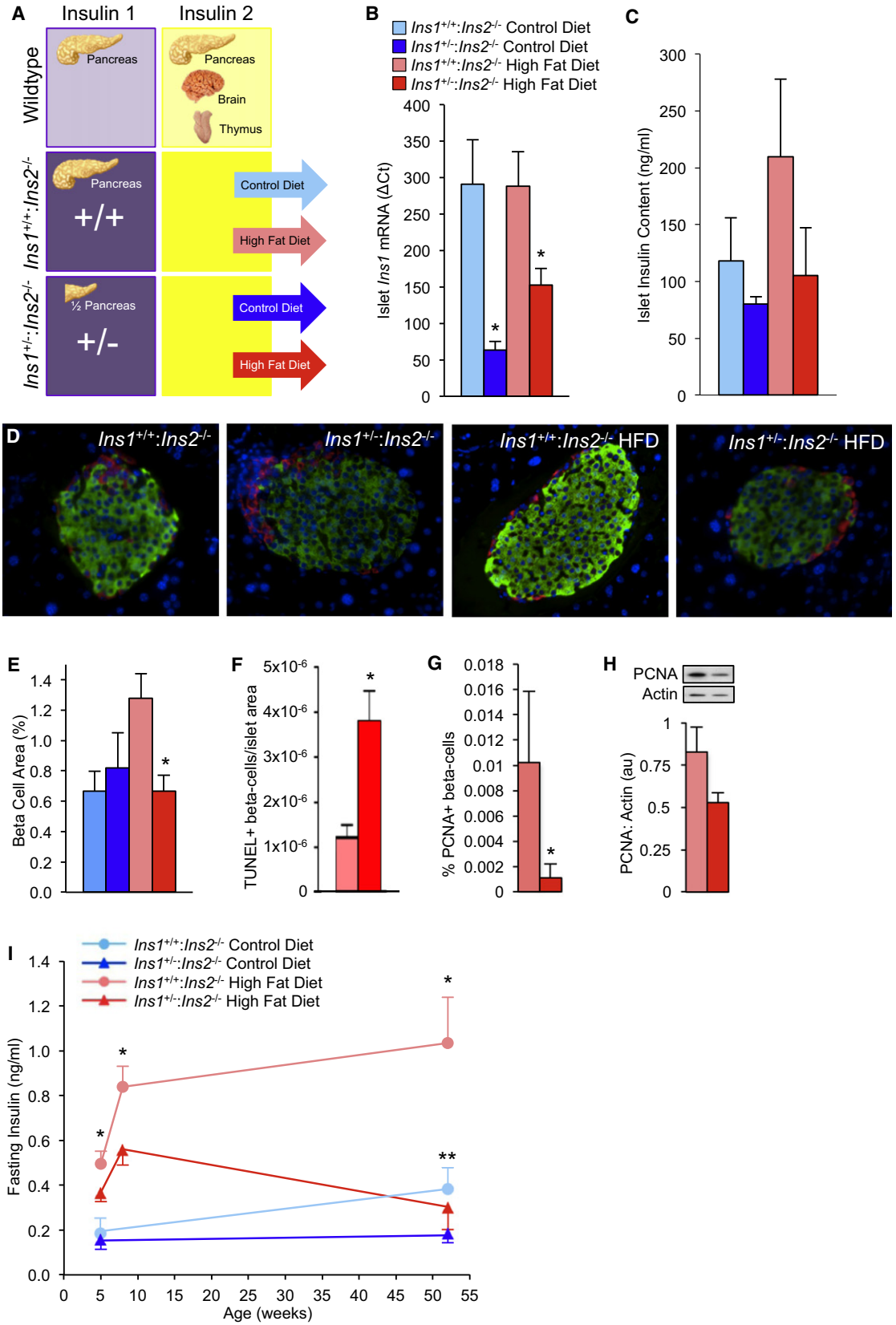
(C) *Ins1* and *Ins2* gene expression assessed by Taqman real-time qPCR in arrayed cDNA from multiple murine brain regions at multiple stages of developing and postnatal brain.

(D) Distribution of neurons expressing insulin protein in the hippocampus of 12-week-old mice (i). Hippocampal section stained without primary antibody (ii). The antibodies were validated with $Ins1^{-/-}$:$Ins2^{+/+}$ and $Ins1^{+/+}$:$Ins2^{-/-}$ mice (see the Supplemental Information).

(E) Close-up view of individual insulin neurons in the rostral cortex.

(F) Colocalization of insulin protein in cell bodies and axons (*green*), with β-galactosidase knocked into the endogenous *Ins2* locus (nucleus; red) in cortical neurons (i), anterior olfactory nucleus neurons (ii), and cerebellar Purkinje neurons (iii) from 12-week-old $Ins1^{-/-}$:$Ins2^{+/-(\beta Gal)}$ mice.

(G) Confocal imaging of morphologically identified neurons showing punctate and reticular C-peptide-2 immunoreactivity (red; ii) within calnexin-positive endoplasmic reticulum (ER; green; iii) and in the somas of 7 day in vitro cultured rat neonatal hippocampal neurons (approximately half of the cells were positive). EGFP transfection was used to outline the boundaries and morphological health of individual neurons (i). Identical results were observed with mouse hippocampal neurons and staining was absent in neurons from $Ins1^{-/-}$:$Ins2^{-/-}$ mice (see the Supplemental Information). Error bars represent standard error of the mean. See also Figures S1 and S2.


A
Insulin 1
Insulin 2
Wildtype
Pancreas
Pancreas
Brain
Thymus
Ins1+/+:Ins2-/-
Pancreas
+/+
Control Diet
High Fat Diet
Ins1+/-:Ins2-/-
½ Pancreas
+/-
Control Diet
High Fat Diet
B
Ins1+/+:Ins2-/- Control Diet
Ins1+/-:Ins2-/- Control Diet
Ins1+/+:Ins2-/- High Fat Diet
Ins1+/-:Ins2-/- High Fat Diet
Islet Ins1 mRNA (ΔCt)
C
Islet Insulin Content (ng/ml)
D
Ins1+/+:Ins2-/-
Ins1+/-:Ins2-/-
Ins1+/+:Ins2-/- HFD
Ins1+/-:Ins2-/- HFD
E
Beta Cell Area (%)
F
TUNEL+ beta-cells/islet area
G
% PCNA+ beta-cells
H
PCNA
Actin
PCNA: Actin (au)
I
Ins1+/+:Ins2-/- Control Diet
Ins1+/-:Ins2-/- Control Diet
Ins1+/+:Ins2-/- High Fat Diet
Ins1+/-:Ins2-/- High Fat Diet
Fasting Insulin (ng/ml)
Age (weeks)

out the possibility that insulin immunoreactivity arose from insulin taken up into specific neurons via endocytosis, we cultured hippocampal neurons in defined insulin-free media. Using an antibody that recognizes mouse C-peptide 2, but not mouse C-peptide 1 (Figure S2C), we identified endoplasmic reticulum (ER) staining in cultured neurons (Figure 1G). The diffuse staining pattern of insulin distribution, also observed with an antibody to fully processed mature insulin, suggests a constitutive release expected for a local trophic factor. Together, these data demonstrate that *Ins2* is produced in the mammalian brain by a subset of neurons, a feature conserved from lower animals (Rulifson et al., 2002). Previous reports of insulin produced in the brain were highly controversial because it was difficult to distinguish low levels of insulin from background signals in the absence of ideal negative controls ($Ins2^{-/-}$ and/or $Ins1^{-/-}$ mice) and positive controls [$Ins1^{-/-}$:$Ins2^{+/-(\beta gal)}$ mice]. The differential expression of two murine insulin genes and the availability of *Ins1* and *Ins2* knockout mice presented a unique opportunity to specifically dissect the role of circulating *Ins1* derived from the pancreas.

Reduced *Ins1* Prevents Diet-Induced β Cell Growth and Fasting Hyperinsulinemia

To test the hypothesis that peripheral, pancreas-derived insulin drives diet-induced obesity, we endeavored to selectively lower circulating insulin by generating mice null for the brain-expressed *Ins2* gene while modulating the gene dosage of pancreas-specific *Ins1* (Figure 2A). It was critical to first test whether removal of one *Ins1* allele in $Ins2^{-/-}$ mice would result in a proportional reduction in *Ins1* mRNA and circulating insulin. Indeed, $Ins1^{+/-}$:$Ins2^{-/-}$ mice exhibited an ~50% reduction in islet *Ins1* mRNA compared to $Ins1^{+/+}$:$Ins2^{-/-}$ mice (Figure 2B). Analysis of insulin secretion and insulin content in islets in young mice revealed some posttranscriptional compensation (Figures 2C and S4). However, by 1 year, insulin immunostaining, fasting circulating insulin, and glucose-stimulated insulin secretion were all markedly decreased in mice with reduced *Ins1* gene dosage (Figures 2D, 2I, and 3A).

Although the $Ins1^{+/+}$:$Ins2^{-/-}$ control mice used in these experiments have been reported to be indistinguishable from wild-type mice on a control diet (Duvillié et al., 1997; Leroux et al., 2001), it was essential to determine whether they would respond to a high-fat diet with a similar hyperinsulinemia and β cell mass increase seen in other control strains (Okada et al., 2007). Relative to chow-fed controls, $Ins1^{+/+}$:$Ins2^{-/-}$ mice fed a 58% fat diet since weaning developed age-dependent, high-fat diet-induced fasting hyperinsulinemia by 7 weeks of age (Figures 2I and S4A). Persistent hyperinsulinemia was dependent on the expression of both *Ins1* alleles.

The fasting hyperinsulinemia in high fat-fed $Ins1^{+/+}$:$Ins2^{-/-}$ mice was associated with an ~2 fold increase in fractional β cell area, compared with mice fed a control diet (Figure 2E). However, in high fat-fed $Ins1^{+/-}$:$Ins2^{-/-}$ mice, β cell area was equivalent to control diet levels, pointing to a critical role for basal circulating insulin in this process (Figure 2E). Analysis of programmed cell death and proliferation in β cells from $Ins1^{+/-}$:$Ins2^{-/-}$ mice suggested that circulating insulin had gene dosage-dependent antiapoptotic and proproliferative autocrine effects in the context of a high-fat diet (Figures 2F–2H). These observations complement studies showing that mice lacking insulin receptors on their β cells fail to increase β cell mass due to defective proliferation and increased apoptosis (Okada et al., 2007). Thus, long-term diet-induced fasting hyperinsulinemia was prevented in $Ins1^{+/-}$:$Ins2^{-/-}$ mice (Figure 2I), which was associated with a lack of diet-induced β cell expansion (Figure 2E). In other words, we generated mice that were genetically incapable of high-fat diet-induced fasting hyperinsulinemia. We were unable to observe high-fat diet-induced hyperinsulinemia or high-fat diet-induced obesity in female $Ins2^{-/-}$ mice regardless of their *Ins1* gene dosage (Figure S3). Male mice were used for all subsequent studies designed to examine the effects of diet on obesity.

Glucose Homeostasis in Mice with Reduced Insulin Gene Dosage

Somewhat surprisingly, we observed only transient differences in fasting glucose between $Ins1^{+/-}$:$Ins2^{-/-}$ and $Ins1^{+/+}$:$Ins2^{-/-}$ mice, despite a modest reduction in glucose-stimulated insulin release (Figures 3A, 3B, and S4B). Significant worsening of glucose homeostasis in $Ins1^{+/-}$:$Ins2^{-/-}$ mice was only observed in a small subset of high fat-fed mice (two mice) during the period of rapid somatic growth around 11 weeks of age and was not observed later in life or on the chow diet (Figure 3C). Insulin sensitivity was generally similar between the genotypes and diets (Figure 3D). Therefore, comparing $Ins1^{+/+}$:$Ins2^{-/-}$ mice and $Ins1^{+/-}$:$Ins2^{-/-}$ mice enabled us to test the effects of genetically reduced pancreatic insulin secretion on obesity, in the absence of sustained hyperglycemia or insulin resistance.

Reduced *Ins1* Gene Dosage Prevents Diet-Induced Obesity

We directly tested the hypothesis that pancreatic insulin hypersecretion is required for diet-induced obesity by tracking body

Figure 2. Reduced Insulin Gene Dosage Prevents β Cell Expansion and Sustained Hyperinsulinemia on a High-Fat Diet

(A) Experimental design to test the role of circulating insulin on diet-induced obesity in the absence of *Ins2*.

(B) qRT-PCR analysis of *Ins1* mRNA in islets from 12-week-old mice (n = 3–5).

(C) Islet insulin content from 30 hand picked islets from 8-week-old mice (n = 3).

(D) Representative staining using anti-insulin (green) and anti-glucagon (red) antibodies in pancreas tissue sections from 12-month-old mice. We found no evidence of islet autoimmunity.

(E) $Ins1^{+/-}$:$Ins2^{-/-}$ mice failed to increase β cell mass in response to a high-fat diet (n = 3).

(F) Cell death was assessed by measuring the number of TUNEL-positive β cells in pancreatic sections from 8-week-old mice (n = 3-5).

(G and H) Proliferation was estimated by quantification of the percentage of PCNA-positive β cells in pancreata from 8-week-old mice (n = 3) and by performance of PCNA immunoblot in islets isolated from 12-week-old mice (n = 4–6).

(I) Age-dependent high-fat diet-induced fasting hyperinsulinemia was prevented in $Ins1^{+/-}$:$Ins2^{-/-}$ mice (n = 6–16).

*Significant difference between high-fat diet (HFD) mice ($p < 0.05$). **Significant difference between control diet (CD) mice ($p < 0.05$). Error bars represent the SEM. See also Figures S3 and S4.

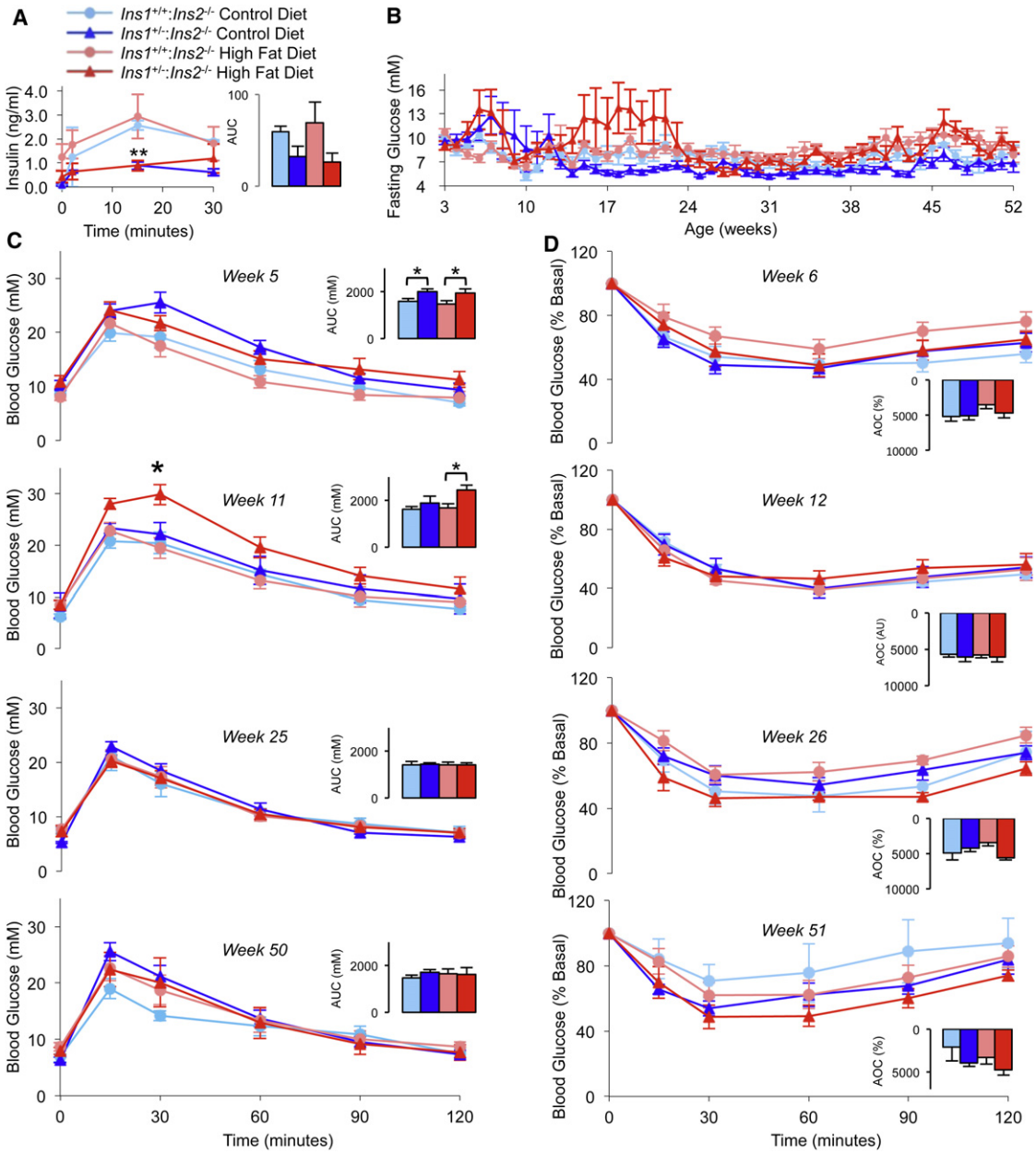


Figure 3. Glucose Homeostasis and Insulin Sensitivity in Mice with Reduced Insulin Gene Dosage

(A) Insulin release in response to intraperitoneal injection of 18% glucose in 53-week-old mice.

(B) Four-hour fasted glucose levels measured weekly over the first year of life.

(C) Intraperitoneal glucose tolerance was impaired in young, but not old *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice. Insets show the area under the curve (AUC).

(D) Insulin tolerance was statistically similar between all groups at all ages studied. Insets show the area over the curve (AOC).

All data are represented as means of at least five male mice in each group at all time points. *Statistical significance ($p < 0.05$). Error bars represent the SEM. See also Figures S3 and S4.

weight of *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ mice and *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice, fed a control diet or an obesigenic high-fat diet (Figure 4A). We observed robust adult-onset obesity in male high fat-fed *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ control mice with all the expected hallmarks, including increased fat-to-lean ratio measured by nuclear magnetic resonance (NMR), increased fat pad weight, increased size of individual adipocytes, and increased inflammation markers in white adipose tissue (Figure 4). In striking contrast, *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice were completely protected from diet-induced adult-onset weight gain for the duration of these 1-year-long experiments (Figure 4A). The high-fat diet-induced increase in epididymal fat pad weight was completely prevented (Figure 4B), resulting in reduced circulating leptin (Figure 4C). Whole-body growth, measured by tibia length, was independent of *Ins1* gene dosage (Figure 4D). Weights of other organs were not statistically different between any of the groups (Figure 4E). Even at ages

prior to the significant divergence of body weight between high fat-fed groups, fasting insulin and body weight were positively correlated between mice within genotypes (Figure S4). Together with the results above, these data suggest that circulating *Ins1* is an adipocyte-specific and β cell-specific growth factor.

Next, we sought to determine the physiological mechanisms accounting for the lack of high-fat diet-induced adiposity in the nonhyperinsulinemic *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice (Figures 4F and 4G). Using indirect calorimetry, we found increased energy expenditure preceding the onset of differences in body weight (Figure 4H). The timing of this observation was important because the normalization of energy expenditure between mice with different levels of adiposity is complicated (Himms-Hagen, 1997). Notably, we did not observe significant differences in food intake, physical activity, or respiratory quotient (Figures 4I–4L). Collectively, these in vivo data suggest that high-fat diet-induced hyperinsulinemia promotes adult adipocyte hypertrophy and nutrient storage. The loss of one *Ins1* allele was sufficient to increase energy expenditure and prevent adiposity in the context of a high-fat diet.

Ins1 Is a Negative Regulator of Uncoupling, Lipolytic, and Inflammatory Genes in White Fat

We predicted changes in energy expenditure and fat cell growth over this long time scale would be associated with stable alterations in gene expression patterns and cellular reprogramming, rather than only acute signaling events. Indeed, while hyperinsulinemic *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ mice exhibited marked adipocyte hypertrophy, white adipose tissue from high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice appeared similar to that from mice fed a control diet (Figure 5A). To determine the molecular mechanisms underlying the protective effects of reduced circulating insulin, we designed a Taqman real-time PCR miniarray of 45 key metabolic, inflammatory, and insulin target genes (see the Supplemental Information for the rationale behind each gene choice). In white adipose tissue, there were few differences between the two groups of mice on the low-fat diet, although insulin receptor mRNA was significantly decreased in *Ins1*$^{+/-}$ mice (Figure 5B). In keeping with the lean phenotype and increased energy expenditure, we observed a gene expression pattern associated with energy expenditure and lipid mobilization, including increased expression of uncoupling proteins and *Pnpla2* (adipose triglyceride lipase), in white adipose tissue from high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice versus high fat-fed *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ mice (Figure 5B). The increase in *Ucp1* gene expression in white adipose tissue isolated from high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice suggested brown adipose tissue-like features similar to what have been observed in other lean models (Leonardsson et al., 2004). *Ppargc1a* (Pgc1α) and *Pparγ*, positive regulators of Ucp1 and energy expenditure, were also upregulated at the mRNA level in white adipose tissue from high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice. Furthermore, we observed downregulation of factors previously shown to promote obesity and adipocyte differentiation in the white adipose of high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice, including the insulin target *Egr2* (Krox20) and *Nrip1* (RIP140), a corepressor that suppresses Ucp1 expression in white adipose tissue (Leonardsson et al., 2004) (Figure 5B). Interestingly, none of the uncoupling proteins were differentially expressed between brown adipose tissue of high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice and high fat-fed *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ mice (Figure S5A). We observed a tendency for a gene expression profile promoting energy expenditure in skeletal muscle from high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice, although these effects were not statistically significant with this sample size (Figure S5B).

We selected a few differentially expressed genes with known roles in insulin signaling and/or adipocyte differentiation for additional analysis at the protein level. Immunocytochemistry showed what appeared to be enhanced Ucp1 protein in white adipose tissue from high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice compared with high fat-fed *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ littermates (Figure 5C). A significant increase in Ucp1 protein levels in high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice was confirmed and quantified by immunoblot of white adipose tissue (Figure 5D). We also observed elevated protein levels of Pparγ and Srebf1/SREBP-1c (Figure 5E), pointing to the upstream transcriptional control of this process by insulin. Immunoblot also confirmed a significant decrease in Egr2 protein in high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice (Figure 5F), mirroring the effects on its mRNA and suggesting a possible "dedifferentiation" of white adipocytes. These quantitative data clearly demonstrate that the pancreas-specific *Ins1* gene, and by extension the circulating insulin hormone, influences the expression of a large number of key metabolic genes in white adipose tissue, specifically in the context of a high-fat diet.

It is well established that obesity and insulin resistance are associated with chronic low-grade inflammation of multiple tissues, including white fat. However, the cause-and-effect relationships between these phenomena and hyperinsulinemia have not been fully established. Our qRT-PCR analysis revealed a broad reduction in inflammatory markers, including *Tnfα* and *Emr1* (F4/80; macrophages) in white adipose tissue from high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice compared with high fat-fed *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ littermates. These data suggest that insulin regulates adipose inflammation either directly (e.g., by modulating immune cells), or indirectly (e.g., via the effects on adipocyte size/stress).

Reduced Pancreatic *Ins1* Protects Mice from Lipid Spillover and Fatty Liver

Ins1$^{+/-}$:*Ins2*$^{-/-}$ mice were protected from elevated circulating free fatty acids when compared to their high fat-fed *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ littermate controls (Figure 6A). In accordance with the lack of lipid spillover, livers of high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice were protected from high-fat diet-induced hepatic steatosis (Figure 6B) and associated stress pathway activation (Figure 6C). The increase in liver triglyceride content in high fat-fed control *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ mice was significantly reduced in *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice, while significant differences in cholesterol were not observed (Figures 6D and 6E). qRT-PCR analysis of liver showed that most of the differences were related to diet (reduced *Ddit3*, *Pparg*, *Ptpn1*, *Il6*, *Igfbp1*, *Egr1*, and *Egr2*; increased *Glut4*, *Srebf1*, *Ppargc1a*, and *Pck1*), rather than between genotypes on the high-fat diet (Figure 6C). There were also notable differences in some inflammation markers in bulk liver tissue (Figure 6C). The reduction in the stress marker *Atf3* (Figure 6C) prompted us to evaluate markers of oxidative stress and lipid peroxidation in this tissue, but the levels of protein carbonyl and 4-hydroxynonenal (HNE) were not different between high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice and their high fat-fed *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ littermates (Figure 6C). Our data support a paradigm whereby

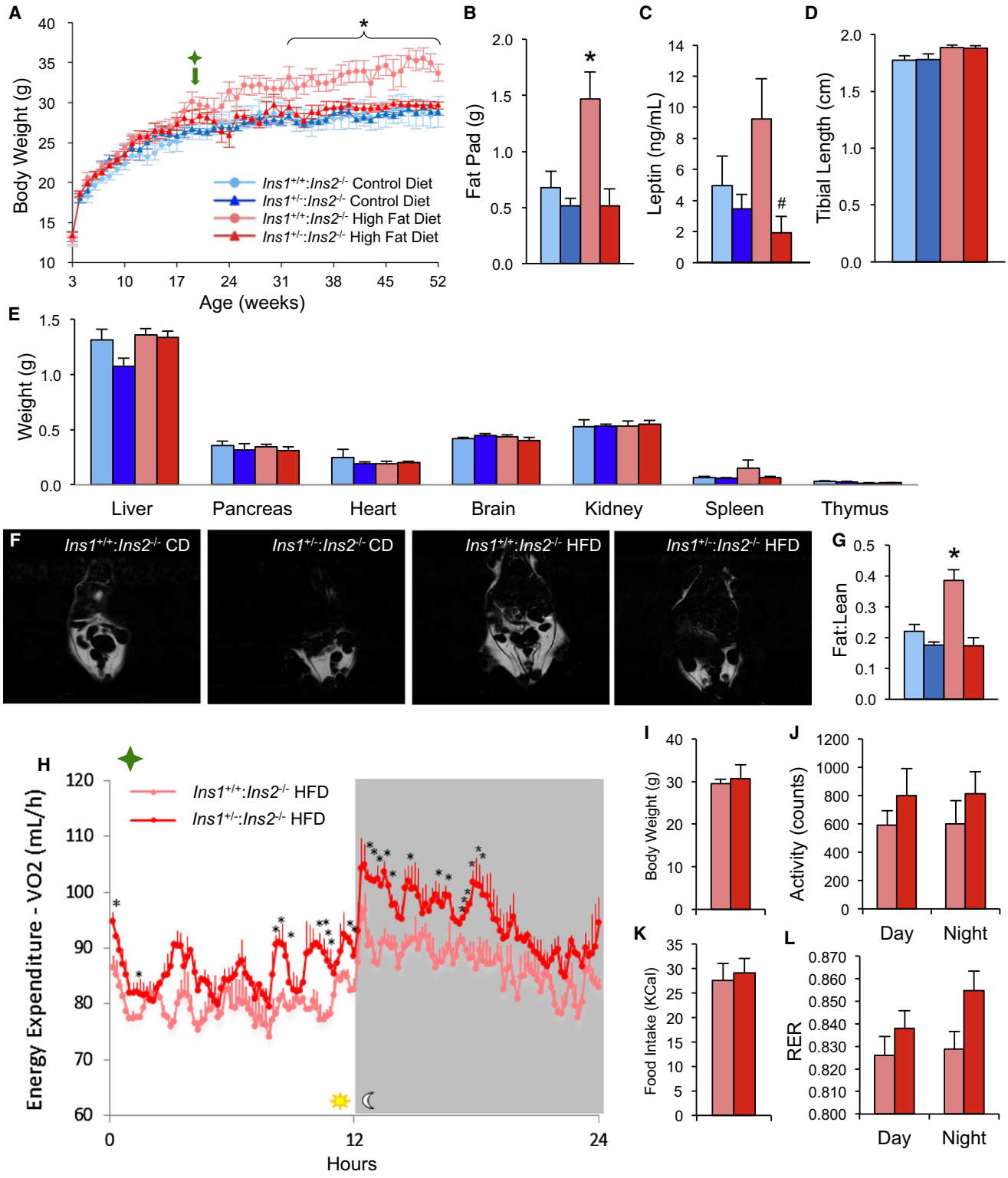


Figure 4. Mice with Reduced Fasting Insulin Are Protected from High Fat-Induced Obesity as a Result of Increased Energy Expenditure

(A) Body weight tracked over 1 year, in multiple independent cohorts (assessed over several years; n = 5–11).

(B) Epididymal fat pad weight in 1-year-old mice (n = 5–11).

(C) Circulating leptin levels (n = 3).

(D and E) Tibial length and organ weight were measured at 1 year as indexes of somatic growth (n = 5–11).

(F and G) NMR spectroscopy (n = 3).

high fat consumption leads to chronic basal insulin hypersecretion (perhaps via direct insulinotropic effects of fatty acids), which then increases adipocyte size and lipid accumulation in adipose tissue. The spillover of free fatty acids subsequently leads to steatosis and ER stress in the liver and other tissues, which may eventually result in pathway- and/or tissue-specific insulin resistance when combined with additional factors (Figure 7). The combination of these factors, initiated by hyperinsulinemia-induced obesity, could predispose individuals to type 2 diabetes.

DISCUSSION

Pancreatic Insulin Hypersecretion Drives Fat Storage and Prevents Fat Burning in Adipocytes

A canon of diabetes and obesity research is that obesity-associated insulin resistance causes hyperinsulinemia because the pancreatic β cells are hyperstimulated to release more insulin, although the physiological mechanism for this hyperstimulation remains unclear since it often occurs prior to hyperglycemia. Our data demonstrate that prevention of high-fat diet-induced hyperinsulinemia through partial ablation of the pancreas-specific *Ins1* gene protects mice from diet-induced obesity and associated complications by upregulating genes that mobilize lipids and increase mitochondrial uncoupling while downregulating genes required for differentiation in white adipose tissue. This would be expected to produce small amounts of heat at the expense of maximal calorie storage in white fat. Specifically, we propose a model for diet-induced obesity whereby hyperinsulinemia normally negatively regulates white adipose tissue *Ucp1* expression, via a gene network involving *Pparγ* and *Nrip1*, to suppress energy expenditure (Figure 7). This idea is consistent with the general anabolic role for insulin and the observation that reducing circulating insulin with diazoxide promotes weight loss in obese mice via an increase in basal metabolic rate (Alemzadeh et al., 2008). Our in vivo data place the pancreas-specific *Ins1* gene upstream of obesity, and establish the causality of circulating hyperinsulinemia in adult fat growth in the absence of insulin resistance (Figure 7A). In our study, increased basal insulin secretion occurred without sustained hyperglycemia. Indeed, both insulin resistance and insulin hypersecretion can occur in normoglycemic humans, preceding obesity and insulin resistance (Genuth et al., 1971; Gray et al., 2010; Ishikawa et al., 1998; Kitamura et al., 2003; Koopmans et al., 1997; Le Stunff et al., 2001; Odeleye et al., 1997). It is notable that early hyperinsulinemia was the strongest predictor of type 2 diabetes in a 24 year study (Dankner et al., 2009). Insulin resistance and obesity can also be uncoupled in human lipodystrophies (Hegele, 2003) and in many mouse models (Brüning et al., 1998; Chen et al., 2010; El-Haschimi et al., 2003; Vijay-Kumar et al., 2010; Wang et al., 2010). Further evidence for the concept that hyperinsulinemia can be a primary factor in the metabolic syndrome in humans can be found in the increased risk of childhood weight gain observed in individuals with elevated pancreatic insulin production caused by inheritance of class I alleles of the *INS* gene (Le Stunff et al., 2001). Genetics and epigenetics likely combine with environmental and dietary factors that stimulate insulin hypersecretion early in life, including in utero, to promote fat growth and eventually complications including type 2 diabetes. The therapeutic potential of drugs that inhibit circulating insulin has previously been demonstrated (Alemzadeh et al., 2008; Alemzadeh et al., 1998; Lustig et al., 2006), but here we provide insight into the molecular mechanism of these clinical observations. Our study provides additional rationale for dietary and therapeutic efforts to partially block insulin hypersecretion or peripheral insulin action in specific tissues to combat obesity. However, insulin levels must always be high enough to sustain glucose homeostasis.

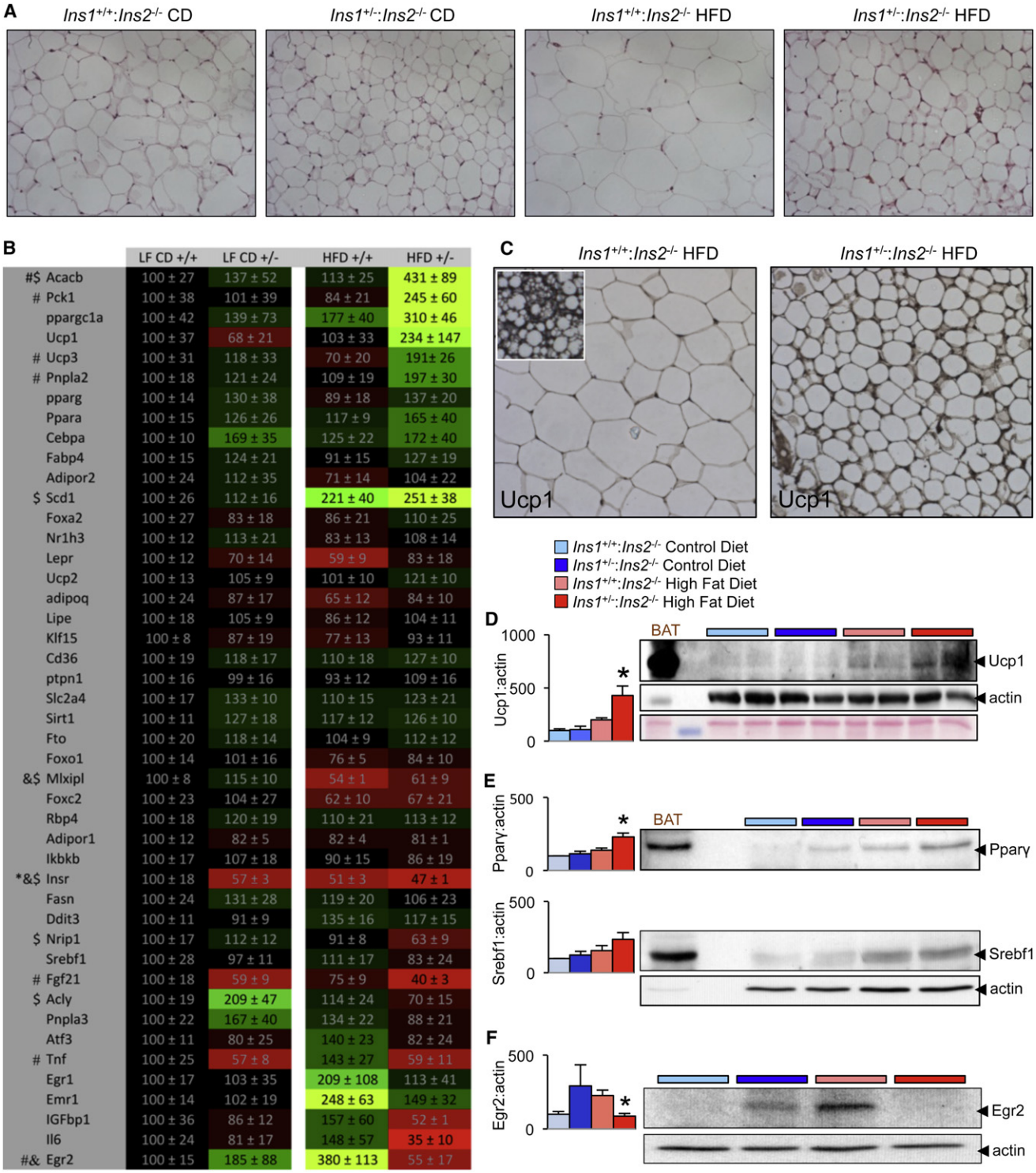
Diet-Induced β Cell Expansion Is Regulated by Insulin In Vivo

Elegant studies have suggested that insulin receptor signaling modulates postnatal β cell mass expansion in response to high-fat diet (Okada et al., 2007), but this concept has remained controversial, in part due to the ability of insulin receptor manipulations to influence IGF1 signaling and to cause compensatory changes in other tissues. The lack of β cell mass "compensation" in $Ins1^{+/-}$:$Ins2^{-/-}$ mice provides direct in vivo evidence that insulin itself is critical for β cell growth and survival under conditions of nutrient stress. Importantly, our results demonstrate that β cell proliferation is induced in the absence of sustained hyperglycemia in vivo. In the past, we have used in vitro cultures of dispersed primary islet cells, a system where insulin and glucose can be clamped independently of each other, to demonstrate that insulin, but not glucose, stimulates β cell proliferation (Beith et al., 2008; Johnson et al., 2006). We have also shown that glucose-stimulated ERK activation is proportionally reduced in islets with reduced insulin gene dosage or treated with somatostatin to block insulin exocytosis (Alejandro et al., 2010; Alejandro et al., 2011), strongly suggesting that glucose modulates β cell fate mainly via local autocrine/paracrine insulin signaling. Indeed, >80% of the effects of glucose on β cell gene expression are lost in β cells with insulin receptor knockdown (Ohsugi et al., 2005). Together, many lines of evidence from multiple groups support the concept that insulin acts via insulin receptors to mediate the compensatory increase in β cell mass and basal insulin release in the context of high-fat diet. This has implications for efforts to increase β cell mass in both type 1 and type 2 diabetes.

Tissue-Specific Roles for Ins1 and Ins2: Relevance to Human INS

Insulin is the most studied hormone in biology, yet the present study provides much needed integrated insight into its localization and physiological functions in vivo. Clinically, insulin insufficiency resulting from a loss of greater than ~80% of functional β cell mass results in diabetes, promoting the prevailing mindset that insulin's roles are almost exclusively positive. Here we show

(H and I) Energy expenditure was measured by indirect calorimetry in high fat-fed mice at 20 weeks of age (green star in A), just prior to differences in weight gain (n = 5).
(J–L) We did not note significant differences in activity, food intake, or respiratory quotient (n = 3–5).
*Statistical significance ($p < 0.05$). Error bars represent the SEM. See also Figures S3 and S4.



	LF CD +/+	LF CD +/-	HFD +/+	HFD +/-
#$ Acacb	100 ± 27	137 ± 52	113 ± 25	431 ± 89
# Pck1	100 ± 38	101 ± 39	84 ± 21	245 ± 60
ppargc1a	100 ± 42	139 ± 73	177 ± 40	310 ± 46
Ucp1	100 ± 37	68 ± 21	103 ± 33	234 ± 147
# Ucp3	100 ± 31	118 ± 33	70 ± 20	191± 26
# Pnpla2	100 ± 18	121 ± 24	109 ± 19	197 ± 30
pparg	100 ± 14	130 ± 38	89 ± 18	137 ± 20
Ppara	100 ± 15	126 ± 26	117 ± 9	165 ± 40
Cebpa	100 ± 10	169 ± 35	125 ± 22	172 ± 40
Fabp4	100 ± 15	124 ± 21	91 ± 15	127 ± 19
Adipor2	100 ± 24	112 ± 35	71 ± 14	104 ± 22
$ Scd1	100 ± 26	112 ± 16	221 ± 40	251 ± 38
Foxa2	100 ± 27	83 ± 18	86 ± 21	110 ± 25
Nr1h3	100 ± 12	113 ± 21	83 ± 13	108 ± 14
Lepr	100 ± 12	70 ± 14	59 ± 9	83 ± 18
Ucp2	100 ± 13	105 ± 9	101 ± 10	121 ± 10
adipoq	100 ± 24	87 ± 17	65 ± 12	84 ± 10
Lipe	100 ± 18	105 ± 9	86 ± 12	104 ± 11
Klf15	100 ± 8	87 ± 19	77 ± 13	93 ± 11
Cd36	100 ± 19	118 ± 17	110 ± 18	127 ± 10
ptpn1	100 ± 16	99 ± 16	93 ± 12	109 ± 16
Slc2a4	100 ± 17	133 ± 10	110 ± 15	123 ± 21
Sirt1	100 ± 11	127 ± 18	117 ± 12	126 ± 10
Fto	100 ± 20	118 ± 14	104 ± 9	112 ± 12
Foxo1	100 ± 14	101 ± 16	76 ± 5	84 ± 10
&$ Mlxipl	100 ± 8	115 ± 10	54 ± 1	61 ± 9
Foxc2	100 ± 23	104 ± 27	62 ± 10	67 ± 21
Rbp4	100 ± 18	120 ± 19	110 ± 21	113 ± 12
Adipor1	100 ± 12	82 ± 5	82 ± 4	81 ± 1
Ikbkb	100 ± 17	107 ± 18	90 ± 15	86 ± 19
*&$ Insr	100 ± 18	57 ± 3	51 ± 3	47 ± 1
Fasn	100 ± 24	131 ± 28	119 ± 20	106 ± 23
Ddit3	100 ± 11	91 ± 9	135 ± 16	117 ± 15
$ Nrip1	100 ± 17	112 ± 12	91 ± 8	63 ± 9
Srebf1	100 ± 28	97 ± 11	111 ± 17	83 ± 24
# Fgf21	100 ± 18	59 ± 9	75 ± 9	40 ± 3
$ Acly	100 ± 19	209 ± 47	114 ± 24	70 ± 15
Pnpla3	100 ± 22	167 ± 40	134 ± 22	88 ± 21
Atf3	100 ± 11	80 ± 25	140 ± 23	82 ± 24
# Tnf	100 ± 25	57 ± 8	143 ± 27	59 ± 11
Egr1	100 ± 17	103 ± 35	209 ± 108	113 ± 41
Emr1	100 ± 14	102 ± 19	248 ± 63	149 ± 32
IGFbp1	100 ± 36	86 ± 12	157 ± 60	52 ± 1
Il6	100 ± 24	81 ± 17	148 ± 57	35 ± 10
#& Egr2	100 ± 15	185 ± 88	380 ± 113	55 ± 17

Figure 5. Molecular Mechanisms Leading to Protection from Diet-Induced Obesity in *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ Mice

(A) Hematoxylin and eosin-stained epididymal fat pad (n = 5–11).

(B) Taqman real-time qPCR quantification of 45 genes in epididymal white adipose tissue from 1-year-old mice. Results are sorted according to the magnitude of the difference between high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice and high fat-fed *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ littermates ($^{\#}p < 0.05$). Genes with a large negative relative expression are colored red; genes with increased expression are green. $^{\$}$Significant difference between diets in the *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice. $^{\&}$Significant difference between diets in the *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ mice. *Significant difference between genotypes on the control diet. Data are from 1-year-old mice (n = 5–11), unless otherwise specified.

(C) Ucp1 immunocytochemistry in white adipose tissue from 1-year-old mice. The inset shows results from the identical staining protocol on brown adipose tissue sections.

that insulin hypersecretion from β cells can be maladaptive. Similarly, elegant studies in flies and worms have demonstrated that deleting insulin genes, or blocking elements of the insulin signaling pathway, dramatically increases life span and prevents diseases associated with adiposity (Broughton et al., 2008; Li et al., 2003). These model systems also clearly indicate that individual insulin-like peptide genes have distinct functions despite signaling through a single receptor (Broughton et al., 2008; Li et al., 2003). In rodents, the *Ins1* and *Ins2* genes are under partially distinct regulation (Alejandro et al., 2011; Hay and Docherty, 2006). Previously, it has also been shown that the mouse *Ins1* and *Ins2* genes have opposing roles on type 1 diabetes incidence in the NOD mouse due to the induction of thymic tolerance by *Ins2* (Fan et al., 2009; Moriyama et al., 2003), but the possibility that they have distinct roles in obesity had not been examined.

In the present study, we elucidated the tissue-specific patterns of *Ins1* and *Ins2* expression. Using multiple approaches, we found that *Ins2* is present in CNS neurons, at both the mRNA and protein levels. Uniquely, we present both negative and positive controls, arguing against qPCR or staining artifacts. We also find distinct active chromatin marks on the *Ins2* gene in the CNS and provide RNA sequencing data. Given the conservation between the rodent *Ins2* gene and the human *INS* gene, we were not surprised to find additional evidence for insulin production in the human brain. In both species, we find robust insulin expression in the hippocampus, pointing to potential roles in learning and memory. It is interesting to note the recent interest in clinical trials using nasal insulin to replace this putative central neurotrophic factor for the treatment of Alzheimer's disease. A complete understanding of the role of *Ins2* produced in the CNS will require brain-specific *Ins2* knockout mice.

We predict that insulin produced locally in specific brain regions will have potent effects on food intake (Hallschmid et al., 2012). Based on recent studies, we expect that some insulin circuits will promote food intake, while others will suppress it, and we predict that distinct populations of insulin neurons will be differentially susceptible to insulin resistance (Könner and Brüning, 2012; Rother et al., 2012). It should be noted that our data do not address what may be an important role of peripheral insulin acting on the brain in the lean phenotype of our mice. Within and outside the brain, we predict that the source of the insulin is critical for its effect on obesity.

Conclusions

We generated mice that were genetically incapable of age-dependent, diet-induced fasting hyperinsulinemia, and thereby demonstrated that circulating insulin plays a causal role in obesity in this model (Figure 7). We identify circulating insulin as a critical regulator of *Ucp1* expression in white adipose tissue and whole-body energy expenditure. We find that increased fat burning in white adipose tissue is associated with reduced lipid accumulation in liver. Our data provide strong rationale to investigate approaches to limit peripheral hyperinsulinemia for the prevention and treatment of obesity in mammals.

EXPERIMENTAL PROCEDURES

Experimental Animals and Glucose Homeostasis

Animal protocols were approved by the University of British Columbia Animal Care Committee in accordance with national and international guidelines. *Ins1*$^{-/-}$ and *Ins2*$^{-/-}$ mice were generated by the group of J. Jami (INSERM, France), and their baseline phenotype described elsewhere (Duvillié et al., 1997). C57Bl6/J mice (Jackson Laboratory, Bar Harbor, ME) were employed for some studies, as indicated. Male mice were used unless otherwise noted. Groups of mice were randomly divided into two diet groups at weaning (3 weeks), and fed a control diet (25% fat) or a high-fat diet (58% fat). Additional details of the diet composition can be found in the Supplemental Information. Body weight, fasting glucose, glucose tolerance, and insulin tolerance were examined after 4 hr fasts according to standard methods we have previously described in more detail (Alejandro et al., 2011). The insulin-positive area was used as an index of β cell mass, as described previously (Alejandro et al., 2011). The cell death was assayed with the Roche TUNEL kit (Alejandro et al., 2011). Serum insulin levels were measured with an ultrasensitive ELISA kit (80-INSMSU-E01; ALPCO Diagnostics, Salem, NH). Leptin levels were measured with mouse leptin ELISA kit (number 90030; CrystalChem, Downers Grove, IL).

In Vivo Metabolic Analysis

PhenoMaster metabolic cages (TSE Systems, Chesterfield, MO) were used for indirect calorimetry, activity, body weight, and food intake studies in accordance with the manufacturer's instructions. After 52 weeks of age, mice were scanned for whole-body fat to lean mass ratio via NMR Spectroscopy at the 7T MRI Research Center (University of British Columbia, Vancouver, Canada). Circulating fatty acids were measured with the Wako NEFA kit.

Gene Expression and Protein Analysis

Mouse and human cDNA arrays, from tissue collected under IRB protocols, were purchased from Origene (Rockville, MD). Gene expression analysis was conducted with custom arrays of Taqman Real-Time PCR Assays (Life Technologies, Burlington, Canada). Immunoblotting and staining were conducted according to standard protocols (Alejandro et al., 2011). Brains were frozen prior to cryosectioning, while all other tissues were fixed in 4% paraformaldehyde. Paraffin block sectioning and hemotoxyn and eosin staining were conducted by the Histology Core at the Child and Family Research Institute (Vancouver, Canada). The following antibodies were used in immunofluorescence studies of neurons and β cells: polyclonal rabbit anti-C-peptide-2 (Millipore), monoclonal mouse anti-mature insulin (Biodesign), and polyclonal guinea pig anti-(pan)insulin (Sigma). Uncoupling protein 1 protein levels were quantified with antibodies from Santa Cruz (catalog number sc-6829), with brown adipose tissue used as a positive control. Images were taken with either an Olympus FV1000 confocal microscope or a Zeiss 200 m microscope equipped with deconvolution software (Slidebook, Intelligent Imaging Innovartions, Denver, CO).

Statistical Analysis

Studies were repeated at least three times. All results are expressed as mean ± SEM (indicated by error bars). Statistical analyses were performed with Graphpad Prism 5 or Microsoft Excel. A student's t test or AVOVA (Tukey's) were used as appropriate. A p value < 0.05 was considered significant.

(D) Immunoblot for Ucp1 in white adipose tissue. Lane 1 is brown adipose tissue (positive control). Equal protein loading was confirmed by β-actin immunoblot and Ponceau staining (pink). Quantification bars are the normalized ratio to β-actin (n = 4–5).
(E) Immunoblots of Pparγ and Srebf1.
(F) Immunoblot analysis of Egr2.
*$p < 0.05$ versus high fat-fed *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ littermates (n = 4–5). Error bars represent the SEM. See also Figure S5.

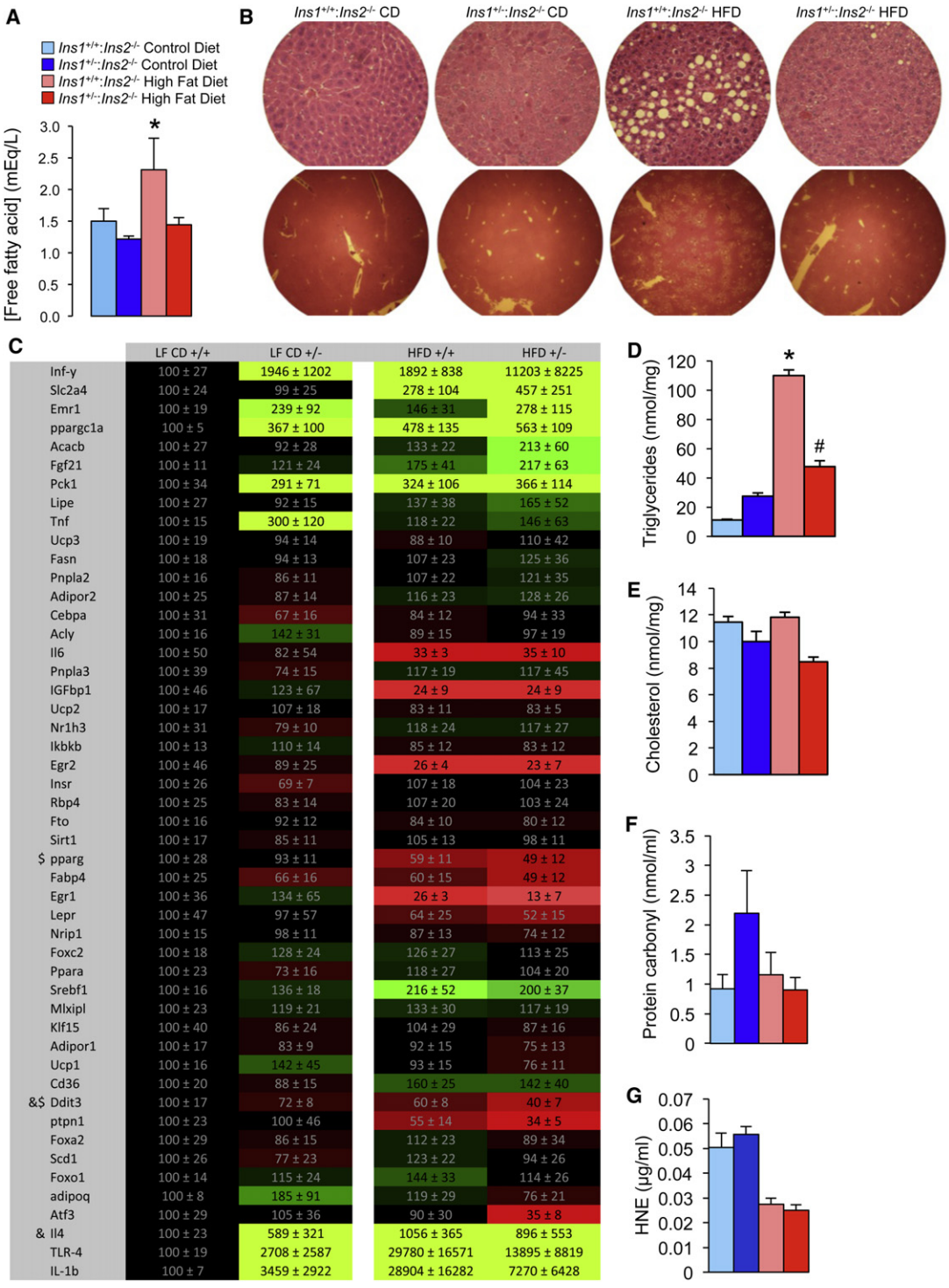


Figure 6. Mice with Reduced *Ins1* Are Protected from Lipid Spill Over, Fatty Liver and ER Stress

(A) Circulating free fatty acids levels.

(B) Low and high magnification of hematoxylin and eosin-stained liver.

(C) Taqman real-time qPCR quantification of 49 genes in liver (n = 3–7 individual mice). Results are sorted as in Figure 3. $^{\#}p < 0.05$ (t test) between high fat-fed *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice versus high fat-fed *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ mice. $^{\$}$Significant difference between diets within the *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice. $^{\&}$Differences between *Ins1*$^{+/-}$:*Ins2*$^{-/-}$ mice versus *Ins1*$^{+/+}$:*Ins2*$^{-/-}$ mice on the control diet.

(D and E) Liver triglyceride and cholesterol measurements (n = 3–5).

(F and G) Markers of oxidative stress, protein carbonyl and 4-hydroxynonenal NHE, were measured in livers (n = 4–6). All data are from 1-year-old mice. Error bars represent the SEM. See also Figure S5.

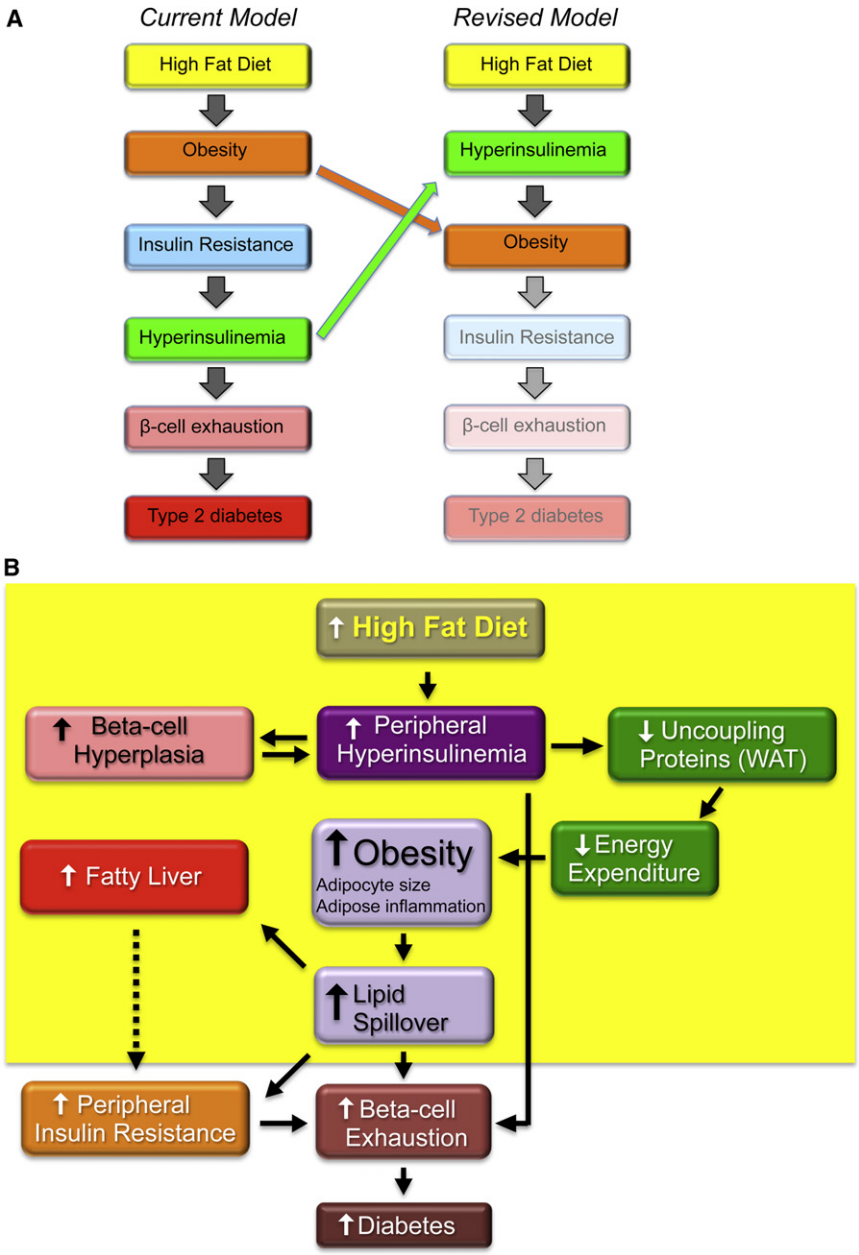


Figure 7. Revisiting the Current Model of Obesity and Type 2 Diabetes

(A) The most widely accepted model of the pathogenesis of obesity and type 2 diabetes posits that a high-fat diet leads to obesity and insulin resistance (there is debate about the relative order and causality of these). In this widely held view, insulin resistance then leads to hyperinsulinemia, which is followed by β cell exhaustion, and then type 2 diabetes. The accepted model is incompatible with our results that put the insulin hypersecretion genetically upstream of obesity.

(B) Our data support a model whereby insulin levels must be kept low to maintain energy expenditure in white adipose tissue via the expression of *Ucp1*. Our data do not address the order of subsequent events after obesity (outside the yellow box), such as insulin resistance and/or type 2 diabetes, since they were not observed in our studies. In other words, the effects of insulin gene dosage on obesity are independent of sustained changes in glucose homeostasis or insulin resistance.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2012.10.019.

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